The EMBO Journal vol.7 no.11 pp.3301 - 3308, 1988

Constitutive transcription and regulation of gene expression in non-photosynthetic plastids of higher plants

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Communicated by J.H.Weil

The plastid genome in higher plants contains >50 genes for rRNAs, tRNAs and proteins for transcriptional and translational functions, besides the genes encoding photosynthetic proteins. Considering the totipotency of most higher plant cells and the differentiation capacity of plastids, it can be inferred that at least the genes for genetic functions must be constitutively expressed in all plant organs, including non-photosynthetic roots, to maintain a basal level of transcriptional and translational activities. To test this hypothesis, transcription, RNA accumulation and polysome formation were analyzed in root amyloplasts, and in plastids from hypocotyls and cotyledons of dark-grown spinach seedlings. The results for 10 representative genes show that they are constitutively transcribed at relative rates which are similar in root amyloplasts and leaf chloroplasts. The differential accumulation of their mRNAs in roots and other non-photosynthetic plant organs is controlled at the post-transcriptional level by a developmental program. Although mRNAs for photosynthetic proteins are detectable in root amyloplasts, some of them are specifically depleted from polysomes relative to mRNAs for ribosomal proteins. This translational discrimination does not result from modifications in splicing or 5'- and 3'-end processing of mRNAs for photosynthetic proteins, since processing is identical in root amyloplasts and leaf chloroplasts. The results support the model of constitutive transcription of the plastid genome, and indicate that the expression of most plastid genes in spinach plants is controlled primarily by post-transcriptional and translational mechanisms.

Key words: plastid/transcription/translation/gene expression/chloroplasts

Introduction

The principal function of plastids in higher plants is the photosynthetic fixation of atmospheric CO_2 in chloroplasts of green tissues, but they can also differentiate into other plastid types to assume specialized physiological functions in non-photosynthetic organs (Kirk and Tilney-Bassett, 1978). For instance, chromoplasts in fruits and certain flower petals develop from chloroplasts (e.g. Rosso, 1968; Spurr and Harris, 1968; Harris and Spurr, 1969; Lichtenthaler, 1970), and accumulate large amounts of carotenoids, which results in the red and yellow colors of these organs (Karrer and Jucker, 1951; Goodwin, 1952). Amyloplasts in roots or in tissue culture cells synthesize starch which is deposited as large granules, and it has been suggested that they may be an essential part of the mechanism of graviperception in root caps (Ophir and Ben-Shaul, 1973). As chromoplasts, amyloplasts or other plastid types can differentiate from photosynthetically active chloroplasts, many (if not all) of these specialized plastids maintain the capability to re-differentiate into chloroplasts. The differentiation capacity of plastids has been postulated (Thomson and Whatley, 1980), and was convincingly demonstrated in early regeneration experiments of single tomato root protoplasts into plants (Norton and Boll, 1954). Considering the totipotency of most higher plant cells, and the differentiation capacity of plastids during plant development, this implies that all plastids must maintain transcription and translation functions for the following reasons. From the complete sequence information for two plant plastid genomes we have learned that, besides genes for photosynthetic proteins, there are >50 genes which encode products for transcription and translation (Shinozaki *et al.*, 1986; Ohyama *et al.*, 1986). These gene products include putative RNA polymerase subunits, rRNAs, a complete set of tRNAs, and several ribosomal polypeptides. Thus, it is necessary that these genes remain in an active configuration to provide the organelle with at least a basal level of transcription and translation activity.

At present there is little information available on the regulation of plastid gene expression in non-photosynthetic tissues. Earlier experiments with amyloplasts in sycamore suspension culture cells have shown methylation of their DNA and undetectable mRNA levels for most plastid genes (Macherel *et al.*, 1986). This, however, may reflect the differentiation state of these cells, which are incapable of forming chloroplasts. In contrast, most plastid genes are still transcribed during chromoplast differentiation in tomato fruit, although mRNAs for photosynthetic proteins decline and the photosynthetic apparatus is disassembled (Piechulla *et al.*, 1985, 1986, 1987; Gruissem *et al.*, 1987). Similarly, the cyclic morphological transformation of chloroplasts into amyloplast-like plastids during the growth cycle of spinach suspension culture cells is accompanied by changes in plastid DNA levels and the differential accumulation of plastid rRNA and the mRNA for the large subunit of ribulose-1,5-bisphosphate carboxylase (Aguettaz *et al.*, 1987). In all of these examples, however, specialized plastids are derived from chloroplasts and, except for tomato chromoplasts, their transcriptional activity has not been determined directly.

Recently Deng and Gruissem (1987) and Mullet and Klein (1987) have reported that the transcriptional regulation of several plastid genes is not a primary control step for the differential accumulation of their mRNAs during light-induced chloroplast development in spinach cotyledons and barley leaves, and during spinach leaf maturation. Instead, the relative transcriptional activities of most genes, which are determined by the strengths of their promoter regions

(Gruissem and Zurawski, 1985; Deng *et al.*, 1987), are approximately maintained despite changes in overall transcriptional activities. Thus, the differential accumulation of plastid mRNAs is controlled by post-transcriptional mechanisms, in which inverted repeat sequences located near the 3′ end of the mRNAs may participate (Stern and Gruissem, 1987). Based on these results and the differentiation capacity of plastids, it could be hypothesized that their genes in general are constitutively transcribed and that post-transcriptional mechanisms regulate the accumulation of their RNAs and the selective translation of mRNAs of transcriptional and translational functions. To test this hypothesis, we have systematically examined plastid gene expression in different plant organs of both dark-grown spinach seedlings and hydroponically grown plants at the transcriptional and post-transcriptional levels. The results support the model that plastid genes for photosynthetic and other proteins are constitutively transcribed in root amyloplast, etioplasts and chloroplasts. The differential accumulation of mRNAs in etioplasts of spinach hypocotyls and cotyledons is regulated by a light-independent developmental program. Root amyloplast transcripts also accumulate, and they are correctly processed at their 5′ and 3′ termini and spliced. However, some mRNAs for photosynthetic proteins are selectively excluded from amyloplast polysomes, suggesting a mechanism to control their expression at the translational level.

Results and discussion

Transcription and differential accumulation of plastid mRNAs in non-photosynthetic organs of spinach plants

The levels of mRNAs for five plastid genes in roots, hypocotyls and cotyledons of dark-germinated spinach seedlings, and roots and young leaves from hydroponically grown spinach were analyzed by molecular hybridizations (Figure 1 and Table I). They include the 16S rRNA, and the mRNAs for the 83-kd chlorophyll *a* apoprotein of photosystem I (*psa*A) (Kirsch *et al.*, 1986), the 32-kd Q_B-binding protein of photosystem II (*psb*A) (Zurawski *et al.*, 1982a), the β- and ϵ-subunits of ATPase (*atp*B/E) (Zurawski *et al.*, 1982b), and the large subunit of ribulose-1,5-bisphosphate carboxylase (*rbc*L) (Zurawski *et al.*, 1981). The RNAs are present in the different organs of both dark- and light-grown spinach plants, including the non-photosynthetic roots, although their levels show significant (up to 900-fold) differences (Table I). mRNAs for photosynthetic proteins have been detected in photosynthetically inactive chromoplasts of tomato fruit as well (Piechulla *et al.*, 1985, 1986), which at least in part results from the transcription of their genes in these specialized plastids (Gruissem *et al.*, 1987). In spinach, plastid RNA levels are lowest in roots, increase in hypocotyls, and are significantly higher in cotyledons and leaves. Since this differential plastid mRNA accumulation occurs in the absence of light or light-mediated phytochrome responses (Tobin and Silverthorne, 1985), we conclude that in dark-grown spinach seedlings organ-specific plastid RNA accumulation is developmentally regulated.

To determine if the increase in RNA levels results from changes in transcription activities and DNA copy number, isolated plastids from the different spinach organs were analyzed by run-on transcription and DNA levels were quantitated by hybridization. The results show that changes

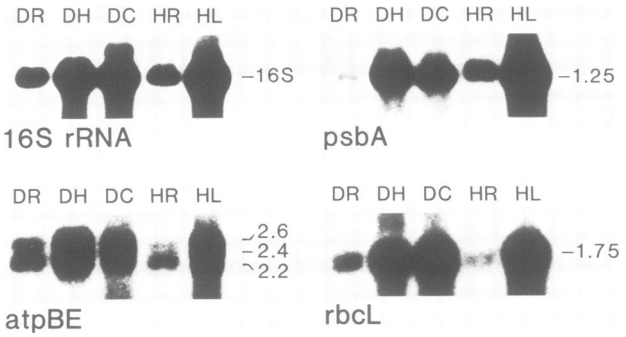

Fig. 1. Organ-specific accumulation of plastid RNAs in spinach. Total RNA from dark-grown roots (DR, 30 μg), hypocotyls (DH, 30 μg) and cotyledons (DC, 2 μg), and total RNA from hydroponic roots (HR, 30 μg) and young leaves (HL, 2 μg) were separated on a 6% formaldeyde/1.2% agarose gel and blotted to nylon membranes. The blots were hybridized with a nick-translated DNA probe (16S *rrn*, 8.7 $\times$ 10^5 c.p.m./ml) or *in vitro* synthesized riboprobes (*psb*A, 3 $\times$ 10^6 c.p.m./ml; *atp*B, 2.5 $\times$ 10^6 c.p.m./ml; *rbc*L, 6 $\times$ 10^5 c.p.m./ml; for abbreviations of plastid genes see legend to Figure 2). RNA extraction and hybridization conditions were identical to previously published procedures (Deng and Gruissem, 1987), except that blots hybridized with the riboprobes were treated with RNase following the final washing step (Deng *et al.*, 1987). X-ray films were exposed for 1 h (16S *rrn*), 2 h (*psb*A), 48 h (*atp*B/E) and 96 h (*rbc*L).

in RNA levels are correlated with an increase in plastid DNA copy number and the overall rate of transcription (i.e. the total incorporation of free nucleotides into hybridizable RNAs per unit of plastid DNA) (Table I). For example, the plastid/nuclear DNA ratio is ~9-fold higher in dark-grown cotyledons as compared with roots, confirming earlier data derived from reassociation kinetics (Scott and Possingham, 1983). The overall rates of transcription in plastids from dark-grown hypocotyls and cotyledons increased ~2.5- and 10-fold respectively, when compared with the rate of transcription in root amyloplasts (Table I). The overall rate of transcription in amyloplasts from roots of hydroponic plants is comparable to that of roots from dark-grown seedlings. Similar studies with tomato fruit chloroplasts and non-photosynthetic chromoplasts indicate a 7- to 10-fold decrease in the overall rate of transcription in chromoplasts during fruit development, but transcription of most genes can still be detected (Gruissem *et al.*, 1987). Together with the decrease in overall transcription activity measured during spinach leaf maturation and barley seedling development (Deng and Gruissem, 1987; Mullet and Klein, 1987), these results suggest that adjustments in the overall rate of transcription may be a general regulatory mechanism for all plastid types in higher plants. It is most likely that changes in the overall rate of transcription result from adjustments of plastid RNA polymerase levels or activity. It is also possible that changes of overall rates of transcription are the consequence of DNA modifications, such as methylation or differences in superhelical density. Based on hybridization experiments with labeled RNA and restriction of plastid DNA with methylation-sensitive enzymes, it has been suggested that methylation of plastid DNA in sycamore tissue culture cells effects the transcription of genes for photosynthetic proteins, but not 16S rRNA (Macherel *et al.*, 1986). However, we were unable to detect methylation of the DNA in spinach root amyloplasts using different methylation sensitive and insensitive restriction enzyme pairs (*Hpa*II/*Msp*I and *Bst*NI/*Eco*RII, data not shown).

Table I. Expression of plastid genes in different organs of spinach plants

	16s rRNA	psbA mRNA	psaA mRNA	rbcL mRNA	atpB/E mRNA	Plastid DNA level	Overall rate of transcription	16s rRNA in polysome
Dark-grown roots	1	1	1	1	1	1	1	1
Dark-grown hypocotyls	4.5	18	7.5	7.9	3.8	1.3	2.5	5.8
Dark-grown cotyledons	67	143	163	349	37	9.2	10	74
Hydroponic roots	0.9	3.7	0.3	0.6	0.7	1.1	1.1	0.8
Hydroponic leaves	85	912	211	383	57	8	30	86

Relative plastid RNA levels were calculated by normalizing the amounts of hybridized labeled probes in RNA blots as shown in Figure 1 to 1 μg of total RNA. Relative plastid DNA levels were analyzed by hybridization of a cytoplasmic rRNA probe and the chloroplast *atp*B probe to Southern blots of total *Eco*RI-digested DNA (Deng and Gruissem, 1987). Overall rates of transcription were determined by calculating hybridizable plastid transcripts synthesized in the 8 min run-on reaction/μg plastid DNA (Deng *et al.*, 1987). Relative levels of plastid 16S rRNA in total polysomes were analyzed by hybridization and normalized to 1 μg of polysomal RNA. All relative levels in the spinach organs were compared to dark-grown roots (arbitrarily set to 1).

Developmentally controlled accumulation of plastid RNAs in spinach is regulated at a post-transcriptional level

The results in Table I also show that superimposed on the general increase of RNA levels in hypocotyl and cotyledons of dark-grown spinach seedlings, individual mRNAs accumulate to significantly different levels. For example, mRNAs from *rbc*L and *psb*A accumulate ~350- and 140-fold in cotyledons of dark-grown seedlings as compared with roots respectively. However, while the relative mRNA levels for *rbc*L, *psa*A and *atp*BE differ by <2-fold in cotyledons of dark-grown seedlings and photosynthetically active leaves, the *psb*A mRNA level increases another 6- to 7-fold. We also note that the *psb*A mRNA accumulates ~4-fold in hydroponic roots compared with roots from dark-grown seedlings, while the level of most other RNAs is similar (see Table I and Figure 1). Comparison of plastid RNA accumulation in roots of comparable developmental stages from dark- and light-germinated seedlings demonstrates, however, that there is no difference in the level of *psb*A mRNA (data not shown). Therefore the higher *psb*A mRNA level in hydroponic roots may indicate differences in plastid mRNA accumulation during root development rather than light effects due to the hydroponic growth conditions. Together the results suggest that plastid RNA levels are established as part of a developmental program in different plant organs, and that light can further accentuate the levels of individual mRNAs.

We have previously reported that changes in overall rates of transcription per plastid genome during light-induced chloroplast development in spinach cotyledons and leaf maturation do not significantly affect the relative transcriptional activities of most plastid genes. We concluded that the differential accumulation of RNAs from these genes is therefore regulated at the post-transcriptional level (Deng and Gruissem, 1987). To examine whether similar regulatory mechanisms are functional in non-photosynthetic tissues as well, run-on transcriptions were performed using isolated plastids from different organs of dark-grown spinach seedlings and hydroponic plants. Labeled run-on transcripts of plastids from roots and hypocotyls of dark-grown spinach seedlings, and from roots of hydroponic plants, were hybridized to 10 plastid genes. These genes encode photosynthetic proteins (*psa*A, *psb*A, *psb*B, *pet*B, *pet*D, *atp*B and *rbc*L) and proteins required for transcriptional and translational functions (*rrn*, *rpl*2 and *rpo*A). Considering the differentiation capacity of plastids, we would expect that at least *rrn*, *rpl*2 and *rpo*A are transcribed in root amyloplasts. In addition, based on the results shown in Figure 1 and Table I, at least those genes encoding photosynthetic proteins for which mRNAs are detectable should also be transcribed in root amyloplasts and plastids of all other organs, although their relative transcriptional activities may vary. However, the relative transcriptional activities of all 10 genes, which are shown in Figure 2 for root amyloplasts as an example, are very similar in plastids from all organs of hydroponic plants and dark-grown seedlings. Moreover, they do not differ significantly from those previously reported for chloroplasts from green cotyledons, young and mature leaves (Deng and Gruissem, 1987). The low transcription activity

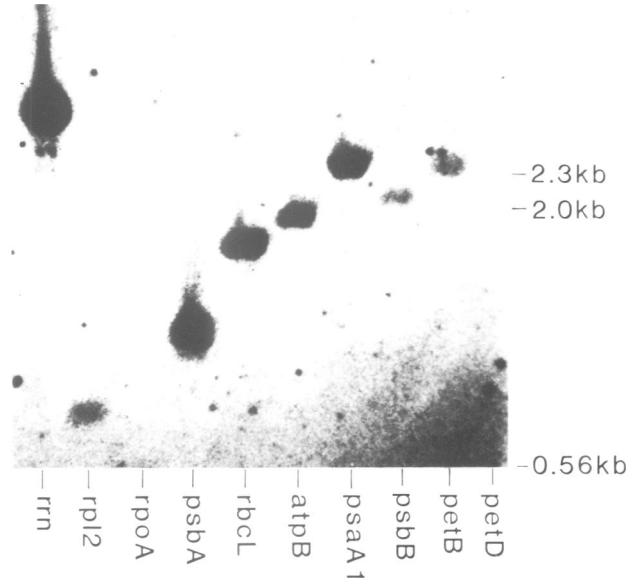

Fig. 2. Relative transcription activities of 10 different plastid genes in root amyloplasts. Stoichiometric amounts of 10 different gene-specific DNA fragments were blotted to a nylon membrane. The membrane was hybridized with run-on transcripts from amyloplasts of hydroponic roots (6×10^4 c.p.m./ml), and the X-ray film was exposed for 9 days. Abbreviations for the plastid genes are: *rrn*, rRNA; *rpo*A, putative α-subunit of the plastid RNA polymerase, *rpl*2, ribosomal protein L2; *psb*A, 32-kd protein or Qb-binding proteins of photosystem II; *rbc*L, large subunit of ribulose-1,5-bisphosphate carboxylase; *atp*B/E, β/ε-subunits of chloroplast ATPase; *psa*A, core polypeptide of photosystem I reaction center; *psb*B, 47-kd polypeptide of photosystem II complex; *pet*B and *pet*D, cytochrome b_6 apoprotein and subunit IV of the cytochrome b_6/f complex respectively. The construction of the specific probes for the above genes has been described previously (Deng *et al.*, 1987).

of the *rpo*A gene in root amyloplasts is consistent with the results obtained for leaf, where *rpo*A is also one of the most weakly transcribed genes.

These results are significant for several reasons. First, they demonstrate that many, if not all, genes on the plastid genome are constitutively transcribed in non-photosynthetic spinach plastids. This excludes the fact that the transcriptional activity observed in root amyloplasts results primarily from transcription of genes encoding proteins for transcriptional and translational functions. Second, the relative promoter strength of several mono- and polycistronic plastid transcription units is maintained in root amyloplasts and in plastids from other spinach plant organs. This supports our model that the differential transcriptional regulation of most plastid genes is less important for the control of their expression (Deng and Gruissem, 1987; Gruissem *et al.*, 1988). Third, the differential accumulation of plastid RNAs in different organs of dark-grown spinach seedlings and hydroponic plant roots indicates that developmentally controlled post-transcriptional regulatory mechanisms are operational both in non-photosynthetic and photosynthetic tissues.

Root plastid rRNA is assembled into polysomes

Besides transcription of the respective genes, the maintenance of the transcriptional and translational machineries in non-photosynthetic plastids would also require translation of the mRNAs for their plastid genome-encoded components. Since the formation of polysomes is usually correlated with translational activity, we first isolated total polysomal RNA from roots and compared this to polysomal RNA from hypocotyl and cotyledons of dark-grown spinach seedlings. As shown in Table I, based on the same amounts of total polysomal RNA, the ratio of plastid 16S rRNA in total polysomes is ~1:6:74 in roots, hypocotyls and cotyledons of dark-grown seedlings respectively. To exclude that the low level of 16S rRNA in the root polysomal RNA fraction results from co-purification of free 16S rRNA or monosomes, isolated root polysomes were analyzed by sucrose density gradient centrifugation (Figure 3A). As shown in Figure 3B, plastid 16S rRNA can be detected in fractions of the sucrose gradient containing monosomes (fraction I), di-, tri- and tetrasomes (fraction II), and pentasomes together with larger polysomes (fraction III). The presence of functional ribosomes in root amyloplasts which can be assembled into polysomes therefore suggests that they are translationally competent. This would be expected, since approximately one third of the plastid ribosomal polypeptides are themselves translated on plastid ribosomes (Posno *et al.*, 1984).

Root amyloplast ribosomes discriminate between individual mRNAs

The constitutive transcription of genes for photosynthetic proteins in spinach root amyloplasts, as well as the accumulation of their mRNAs, suggests that their expression is controlled at a different level. Since amyloplasts do not assemble a functional photosynthetic apparatus, it is possible that synthesis of proteins for photosynthetic complexes is regulated at the translational and/or post-translational level, for example, by exclusion of their mRNAs from polysomes or rapid turnover of the translated protein. On the other hand, we would expect that, for example, mRNAs for plastid-encoded ribosomal proteins are present on polysomes. To address this problem, we first examined if the translation

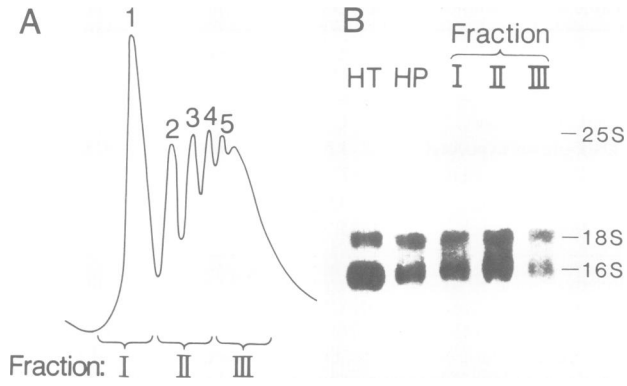

Fig. 3. Distribution of plastid 16S rRNA in spinach root polysomes. (**A**) Sucrose gradient profile of total polysomes from hydroponic roots. The numbers above each peak indicate the number of ribosomes associated with mRNAs. The three fractions pooled for RNA analysis are indicated by roman numbers. (**B**) Hybridization of a spinach plastid 16S *rrn* probe to the three polysomal fractions from hydroponic roots. Hybridization to 6 μg each total (HT) RNA and total polysomal RNA (HP) from hydroponic roots are shown as a control. The band labeled 18S most likely represents cross-hybridization of the plastid *rrn* probe to either 18S cytoplasmic or mitochondrial rRNA.

system in root amyloplasts can discriminate between mRNAs specific for photosynthetic proteins and other mRNAs. We analyzed the distribution of five mRNAs between the polysomal and total RNA fractions (Figure 4). These included the mRNA for the 32-kd quinone-binding protein of PSII (*psb*A), the large subunit of ribulose-1,5-bisphosphate carboxylase (*rbc*L), the cytochrome b_6/f complex subunit IV (*pet*D), and two ribosomal polypeptides (*rps*16 and *rps*19, encoding polypeptides S16 and S19 of the 30S ribosomal subunit respectively). The relative levels of these mRNAs in total and polysomal RNA fractions were compared using quantitative S1 nuclease protection analysis.

The results in Figure 4 show that the mRNAs of two photosynthetic genes, *psb*A and *rbc*L, are found at 6-fold lower concentrations in polysomal than in total RNA. In contrast, mRNAs for the ribosomal polypeptides S16 and S19 are at similar levels or 2-fold enriched respectively in the polysomal RNA. Interestingly, the mRNA of the *pet*D gene is also enriched ~2-fold in polysomal RNA. For *psb*A, the band marked "−86" represents the protected DNA fragment that extends from the *Pst*I site in the coding region to the transcription start site of the mRNA (Zurawski *et al.*, 1982a). The bands for *rbc*L marked "−180" and "−65" designate the DNA fragments that result from protection by the primary and processed transcripts respectively from their 5′ ends to the *Pst*I site in the coding region (Zurawski *et al.*, 1981; Mullet *et al.*, 1985). Quantitation of the protected labeled DNAs indicates that their levels are ~20-fold lower with polysomal RNA relative to total RNA. However, since S1 analysis was performed with equal concentrations of RNA, and plastid 16S rRNA is ~3-fold lower in polysomal RNA compared with total root RNA (see Materials and methods), we conclude that *psb*A and *rbc*L mRNAs levels in polysomal RNA are reduced by at least 6-fold. For *rps*16, a 183 *Xho*I−*Xba*I fragment was used which contains 114 bp of exon sequence and 69 bp of intron sequence (X.-W.Deng and W.Gruissem, unpublished results). The two protected fragments after S1 digestion (114 and 183) therefore represent the spliced and unspliced *rps*16 mRNA. The

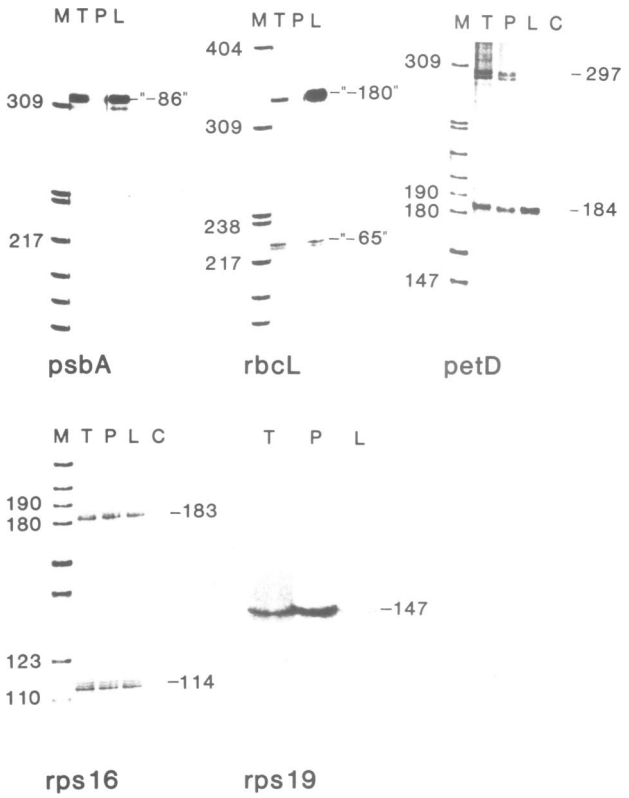

Fig. 4. Relative levels of mRNAs in total RNA and polysomal RNA in hydroponic spinach roots analyzed by S1 nuclease protection assays. For *psb*A, *rbc*L and *pet*D, 35 μg of total (**lane T**) and polysomal (**lane P**) RNA, and 50 ng of chloroplast RNA (**lane L**) were hybridized to excess labeled DNA probes (see Materials and methods). For *rps*16 and *rps*19, 35 μg of total RNA (**lane T**), 100 μg of polysomal RNA (**lane P**), and 1 μg of chloroplast RNA (**lane L**) were added to the S1 reaction. **Lane C** in panel *pet*D and *rps*16 is a control reaction with tRNA only, and **lane M** is a labeled *Hpa*II digest of pBR322 DNA. The level of mRNAs in total and polysomal RNA were calculated from the amount of protected DNA probes by scintillation counting of the radioactivity in each band. Since the *rbc*L mRNA also has a processed 5′ end, the radioactivities from each protected fragment were combined. For *pet*D and *rps*16, only the protected fragments generated from the spliced mRNAs (184 and 114 respectively) were used for the calculations. The numbers to the right of each panel indicate the lengths of the protected fragments. For *rbc*L and *psb*A, the numbers indicate the size of the protected fragment from the 5′ end of the transcripts to the translation start sites.

protected band for *rps*19 (147) represents the mRNA from the 3′ end of the transcript to the *Bal*I restriction site in a 275-bp *Bal*I−*Tag*I DNA fragment (Zurawski *et al.*, 1984). For both *rps*16 and *rps*19 quantitative protection analysis was carried out with total and polysomal RNA concentrations containing equal amounts of plastid 16S rRNA. Thus, the amounts of mRNAs for both ribosomal proteins are similar or higher in polysomal RNA relative to total RNA. Similarly, for *pet*D the two bands at 184 and 297 result from protection of a 297-bp *Bam*HI fragment by spliced and unspliced *pet*D mRNA (Heinemeyer *et al.*, 1984; X.W.Deng and W.Gruissem, unpublished results). Compared with *psb*A and *rbc*L, *pet*D mRNA is clearly enriched in root polysomal RNA.

Taken together, these results suggest that root amyloplasts discriminate among different mRNAs to allow the preferential translation of mRNA species for housekeeping functions in non-photosynthetic tissues. Alternatively, this may simply reflect differences in affinities between ribosomes and ribosome-binding sites of specific plastid mRNAs, which may become apparent if ribosomes are limiting in amyloplasts. If *psb*A and *rbc*L mRNAs have weak ribosome binding sites, their translational discrimination could be overcome by increasing their levels relative to other mRNAs in photosynthetic tissues or in dark-grown organs which will become photosynthetically active. This would be consistent with the results shown in Table I and Figure 4, and the distribution of leaf chloroplast mRNAs in total RNA and polysomes. In spinach leaves the levels of *rbc*L and *psb*A mRNAs in total RNA are ~900- and 380-fold higher relative to root amyloplasts (Table I). In addition, quantitation of *rbc*L and *psb*A mRNA levels in leaf polysomal RNA, based on equal concentrations of plastid 16S rRNA, indicates that their levels relative to total RNA are only reduced 2-fold, as compared with their 6-fold lower level in root polysomal RNA. These results do not exclude, however, a mechanism in which specific translation initiation factors are required for the light-dependent assembly of *rbc*L and *psb*A mRNAs into polysome complexes, as was suggested for these transcripts in barley leaves (Klein *et al.*, 1988).

The polysomal association of mRNAs for ribosomal proteins in root amyloplasts supports our concept that components of the transcription and translation apparatus are constitutively synthesized in non-photosynthetic plastids. The prevalence of *pet*D mRNA in root polysomal RNA is noteworthy and suggests that it is translated. Although it is currently unknown if a functional cytochrome b_6/f complex is present in root amyloplasts, it has been documented that the plastid genome in higher plants encodes a set of actively transcribed genes whose sequence is similar to the human mitochondrial NADH reductase genes (Matubayashi *et al.*, 1987). Thus, it is reasonable to speculate that in root amyloplasts a functional cytochrome b_6/f complex may form an electron transport chain with NADH reductase to provide an energy-generating system.

Plastid transcripts are correctly processed in spinach roots

Chloroplast mRNAs are subject to extensive post-transcriptional processing, including cleavage of polycistronic transcripts, 5′- and 3′-end processing and splicing of introns (for review see Gruissem, 1988). Thus it is conceivable that differences in the processing of amyloplast mRNAs may change their capability to initiate translation. In Figure 4, the protected fragments detected for *psb*A ("−86"), *rbc*L ("−65" and "−180") and *rps*19 (147) already indicate that their mRNAs have identical 5′ or 3′ termini in amyloplasts and leaf chloroplasts. It should be noted that the *rps*19 mRNA is cleaved from a polycistronic RNA precursor, in which there are other transcripts proximal and distal to *rps*19 (Zurawski *et al.*, 1984). The results in Figure 5 demonstrate that the 5′ ends of the *atp*B/E mRNA, and the 3′ ends of the *psb*A and *rbc*L mRNAs, are also identical in roots and leaves. For *rbc*L and *atp*B/E, additional 5′ ends are formed in chloroplasts by processing of the primary transcript. The 520-bp *Eco*RI−*Pst*I fragment spanning the *rbc*L transcription start site also detects the processed mRNA at "−65". The 1038-bp *Sau*96I−*Pst*I DNA fragment containing upstream sequences of *atp*BE (Zurawski *et al.*, 1982b) detects all four 5′ ends that have been reported for the *atp*BE

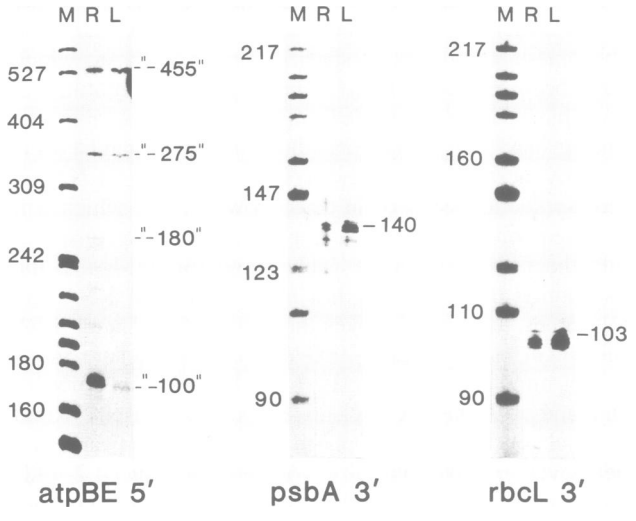

Fig. 5. Comparison of 5′ and 3′ ends of plastid mRNAs in spinach roots and leaves. For the *atp*BE 5′ ends, a 1038-bp *Sau*96I−*Pst*I DNA fragment (Mullet *et al.*, 1985) was end labeled and hybridized to 100 μg root RNA (**lane R**) or 1 μg leaf chloroplast RNA (**lane L**). The four *atp*BE 5′ termini are indicated relative to the *atp*BE translation start site. To compare the 3′ ends of *psb*A and *rbc*L mRNAs, a 320-bp *Xba*I−*Hin*dIII fragment (Zurawski *et al.*, 1982a) and a 245-bp *Eco*RI−*Bam*HI fragment (Zurawski *et al.*, 1981) respectively, were end labeled and hybridized to 100 μg root RNA (**lane R**) and 100 ng leaf chloroplast RNA (**lane L**). The estimated sizes (nt) of the 3′ ends are indicated. **Lane M** is a labeled pBR322−*Hpa*II DNA size marker.

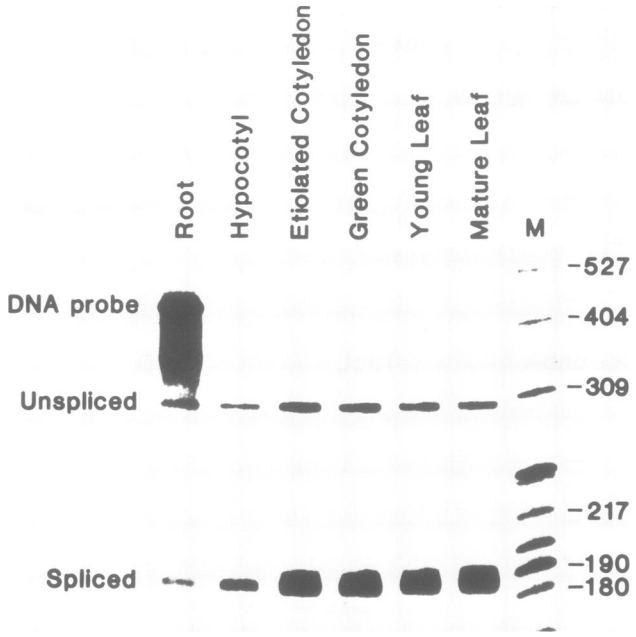

Fig. 6. Ratios of intron-containing and spliced *pet*D mRNA in different organs of spinach. A labeled single-strand DNA probe for *pet*D (see Figure 2) was hybridized to total RNA of roots (120 μg), etiolated hypocotyls (45 μg), etiolated cotyledons (26 μg), green cotyledons (21 μg), young leaves (21 μg) and mature leaves (21 μg). The signals corresponding to the full-length DNA probe, unspliced mRNA protection and spliced mRNA protection are indicated. **Lane M** is a labeled pBR322−*Hpa*II DNA size marker. RNA samples were precipitated twice with 2 M LiCl to eliminate plastid DNA contamination.

transcript (Mullet *et al.*, 1985). The ratio of the transcripts with a processed 5′ end to the unprocessed transcripts is greater in root amyloplasts than chloroplasts for both *rbc*L and *atp*BE. Since mRNA species with both processed and unprocessed 5′ ends are associated with chloroplast polysomes (X.-W.Deng and W.Gruissem, unpublished results), it is unlikely that 5′-end processing is a prerequisite for or prevents translation initiation.

The analysis of 5′ and 3′ ends in amyloplast mRNAs indicates that transcription initiation and processing sites are used which are identical to those in leaf chloroplasts. Figure 4 also demonstrates that *pet*D and *rps*16 mRNAs are spliced at identical sites in roots and leaves, but the relative amount of spliced compared with unspliced mRNAs may vary in amyloplasts and chloroplasts. To examine the accumulation of intron-containing and spliced mRNAs in different organs and plastid types of spinach in more detail, we used the *pet*D transcript as an example in quantitative S1 nuclease protection experiments (Figure 6). As expected, the labeled single-strand DNA probe detects the 297 and 184 nt *pet*D RNA fragments, which represent the spliced and unspliced *pet*D mRNA, respectively. While the ratio of these two RNAs is ~1:1 in root amyloplasts, the ratio of the unspliced to the spliced mRNA increases to 1:7 in illuminated cotyledons and 1:9 in mature leaves. These results indicate that the ratio of spliced to unspliced *pet*D RNA increases with the photosynthetic competence. The data cannot distinguish if this is due to differences in the splicing efficiency of *pet*D mRNA in the different organs, or reflects an increased stability of the spliced *pet*D mRNA in chloroplasts.

Conclusion

Taken together, our results demonstrate that correct transcription and processing of several mRNAs, including those for photosynthetic proteins, also exists in non-photosynthetic plastids. They provide evidence that most likely all genes on the plastid genome in higher plants are constitutively transcribed in all organs. As during chloroplast development and maturation in spinach leaves (Deng and Gruissem, 1987), the reduced overall rate of transcription in root amyloplast affects most plastid genes similarly and does not significantly change their relative transcriptional activities. Together with the capability for transcript processing, splicing, and polysome formation in root amyloplasts, this suggests that transcriptional, post-transcriptional and translational processes are all functional in specialized plastids. Our data therefore strongly support the model that plastids in higher plants maintain basic genetic functions in non-photosynthetic tissues, which is consistent with their competence to redifferentiate into other plastid types.

Our data further support previous studies that the expression of genes for photosynthetic proteins is regulated by post-transcriptional and translational mechanisms during plant development. Several studies have already indicated that plastid gene expression can be regulated at the translational or post-translational level (Fromm *et al.*, 1985; Hermann *et al.*, 1985; Berry *et al.*, 1986; Klein and Mullet, 1986; Gamble *et al.*, 1988). Recently it was demonstrated that transcripts for *psa*A/B and *psb*A are associated with polysomes in dark-grown barley seedlings, but their proteins cannot be detected, suggesting that their synthesis is arrested on polysomes (Klein *et al.*, 1988). The discrimination of the translation apparatus against specific mRNAs for some photosynthetic proteins that we observe in root amyloplasts,

however, appears to differ from the above-described translational regulation, and may indicate a novel translational control mechanism.

Materials and methods

Plant material

Spinach (*Spinacia oleracea* cv. Marathon hybrid) was grown hydroponically under greenhouse conditions. Leaves and roots were harvested immediately before use. For dark-grown plants, spinach seeds were planted in sterile soil, and seedlings were grown in a completely dark growth chamber at 25°C for 6 days. Cotyledons, hypocotyls and roots of dark-grown spinach seedlings were dissected under weak green light and used immediately.

Plastid isolation and run-on transcription

Isolation of chloroplasts from leaves and etioplasts from cotyledons, and run-on transcription assays were performed essentially as described previously (Deng and Gruissem, 1987). The isolation procedure for plastids from non-photosynthetic organs followed a modified protocol for the isolation of etioplasts (Deng and Gruissem, 1987). Briefly, ~10–30 g of spinach roots or hypocotyls were rinsed with 2 vol of 1 × GM (1 mM sodium pyrophosphate, 50 mM Hepes, pH 6.8, 0.33 M sorbitol, 10 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA), blot dried, and homogenized in 120 ml of 1 × GM in a Waring blender at high speed for 6–8 s. The homogenate was filtered through four layers of sterile miracloth. The filtrate was centrifuged at 250 *g* for 1 min to remove debris. The supernatant was centrifuged at 3000 *g* for 1 min. The plastid pellets were resuspended in 30 ml of 1 × IC (1 mM sodium pyrophosphate, 50 mM Hepes, pH 7.9, 0.33 M sorbitol, 10 mM dithiothreitol), and centrifuged at 3000 *g* for 1 min. The final plastid pellets were resuspended in 50 μl of 1 × IC. Run-on transcription and hybridization procedures, determination of plastid DNA content and trichloroacetic acid precipitations followed published procedures (Deng and Gruissem, 1987; Deng *et al.*, 1987).

Analysis of polysomal mRNAs

The procedure for isolation of polysomes was modified from Jackson and Larkins (1976). Tissues were frozen in liquid N_2 and ground. The tissue powder was resuspended in 10 ml isolation buffer (200 mM sucrose, 10 mM dithiothreitol, 25 mM EGTA, 1% Triton X-100, 50 mM Tris–HCl, pH 8.5, 50 mM KCl, 20 mM $MgCl_2$ and 50 μg/ml chloramphenicol) and homogenized. After filtration the homogenate was centrifuged at 12 000 *g* for 10 min. Polysomes were pelleted from the supernatant through a layer of 1.5 ml 2 M sucrose (in 50 mM Tris–HCl, pH 8.5, 100 mM KCl, 10 mM $MgCl_2$) at 200 000 *g* for 90 min at 4°C. Polysome pellets were either subjected to routine RNA extraction or fractionated on sucrose gradients according to Jackson and Larkins (1976). Polysomal RNA from the collected fractions was extracted and precipitated. RNA blotting, hybridization probes and hybridization conditions were essentially as described in Figure 1. For the quantitation of mRNA levels in total and polysomal RNA, equal amounts of labeled single-strand DNA probes were mixed with appropriate amounts of RNA samples and subjected to S1 analysis (Stern and Gruissem, 1987). Yeast tRNA was added to each reaction to adjust the RNA in the reaction mixture to 100 μg. For *psb*A and *rbc*L, a 397-bp *Bgl*II–*Pst*I fragment (Zurawski, 1982a; 4 × 10^5 c.p.m.) and a 520-bp *Eco*RI–*Pst*I fragment (5.5 × 10^4) respectively, were 5′ labeled at the *Pst*I sites. For *pet*D and *rps*16, a 297-bp *Bam*HI fragment (Heinemeyer *et al.*, 1984) and a 183-bp *Xho*I–*Xba*I fragment (Zurawski *et al.*, 1982b), which covers 184- and 114-bp exon sequences, and 123 and 69-bp intron sequences respectively, were inserted into the Bluescribe M13 KS+ vector. For *rps*19, a 275-bp *Bal*I–*Tag*I fragment (Zurawski *et al.*, 1984) was inserted into the pUC119 vector. Single-strand DNAs were isolated, annealed to primer DNA, and used as templates to synthesize the single-strand DNA probes using Klenow in the presence of 200 μM dATP, dGTP, dTTP and 100 μCi [α-^{32}P]dCTP (3000 Ci/mmol; Amersham). After restriction enzyme digestion, the synthesized DNA strands with the attached primer were purified and used as S1 probes.

S1 protection analysis

The S1 protection analysis followed a procedure as described previously (Stern and Gruissem, 1987) and the changes are indicated in the figure legends.

Acknowledgements

We thank Drs Alice Barkan and David Stern for critical reading of the manuscript, and Ning Wei for her help in dissecting the dark-germinated spinach seedlings. X.-W.D. was supported by a University of California Berkeley fellowship. This work was supported by the US Department of Energy (ER13375).

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Received on July 8, 1988